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1. Your reference — GWS\20873

2. Patent application number *(The Patent Office will fill in this part)* — **9904773.0** — 2 MAR 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Microbiological Research Authority
CAMR
Porton Down
Salisbury
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Great Britain

Patents ADP number *(if you know it)* 6566160001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Culture of mycobacteria

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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London WC1X 8AL

Patents ADP number *(if you know it)* 1081001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Signature Mathys & Squire Date 1 March 1999

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CULTURE OF MYCOBACTERIA

The present invention relates to a method of culture of mycobacteria and also to a growth medium therefor.

Health risks associated with mycobacteria have been known for many years and diseases caused by some species are responsible for major global healthcare problems.

The emergence of antibiotic resistant strains of Mycobacteria responsible for tuberculosis (TB) have led to increasing numbers of deaths in those contracting the infection. A TB vaccine based on an attenuated strain of *M.bovis* (BCG) has been available for several years, but protection is restricted to particular ethnic groups, for reasons that are unknown. Mycobacteria have also been associated with several other conditions such as Crohn's Disease.

In addition, there are indications that inoculation with products derived from some mycobacterium species can mediate changes in immune responses which have beneficial effects. Thus, products of this type are currently undergoing evaluation to investigate their therapeutic usefulness against a range of conditions, including TB and cancers.

A major problem associated with the study and production of pharmaceutical products based on mycobacteria is the difficulty associated with bacterial growth. Conventional methods involve growth on solid agar slopes and, consequently, manufacturing products using this type of approach is both labour intensive and costly. These processes are poorly defined leading to batch variation.

Development of improved culture processes have made only limited progress with problems of relatively slow growth rate and bacterial aggregation. Batch culture processes have been associated with apparent loss of virulence, and/or essential components.

The majority of studies to date have grown *M.tuberculosis* as surface pellicles or as agitated dispersed cultures over long periods. Media used have been relatively simple containing a carbon source, nitrogen and buffered salts together with trace elements and were designed to provide high yields free of macromolecular medium components. Glycerol is considered the essential carbon source to ensure copious growth.

Media for culture of tubercle bacilli is described by Dupos et al in AM.REV.TUBERC. volume 56, 1947, pp334-345. A growth medium referred to as "tween-albumin" medium was used, containing 0.01-0.05 percent tween 80 and from 0.5-1.0 percent albumin. This growth medium has hitherto been the standard growth medium used in this field. Wayne L.G. in Infection and Immunity, Sept.1977, pp528-530 used this same medium and reported a mean generation time of 17-18 hours for *Mycobacterium tuberculosis*. Lowrie et al, in Journal of General Microbiology, Volume 110, 1979, pp431-441 used a concentrated version of the same medium. A review by Wayne L.G in Tuberculosis: Pathogenesis, Protection, and Control, published by the American Society for Microbiology in 1994, pp73-83 also describes how most published work in this area has employed 0.02 percent tween 80 and 0.5 percent bovine serum albumin, the albumin being used to protect the bacillus from toxic effects of traces of oleate released from the tween 80. Youmans and Youmans reported in 1959 that 0.05 per cent Tween 80 slowed mycobacterial growth noticeably.

Another problem with existing culture methods and culture media is that the mean generation time, or doubling time, of the bacillus is rather long. For commercial production of mycobacteria it would be desirable to reduce the doubling time so that a greater yield of bacillus and bacillus products, such as enzymes, may be obtained. Typically, it is found that a culture period of at least two or three weeks is necessary until the culture may be harvested to yield any useable volume of products.

Wayne L.G, who has published widely in this area, has reported that growth of mycobacteria in detergent - containing medium results in diminution of virulence, a serious disadvantage.

It is also found that the existing culture methods of mycobacteria produce rather low yields. Lowrie et al obtained yields of around $8x10^8$ bacteria ml^{-1}, but only at very low dilution rates of $0.016h^{-1}$, indicating a doubling time of about 43 hours. Wayne describes a culture having a quicker doubling time, but only at cell densities in the region of $4x10^7$ CFU ml^{-1}. It would accordingly be desirable to provide a culture having a reduced doubling time whilst maintaining high bacterial densities.

It is desirable to produce virulent mycobacteria at increased yields whilst maintaining virulence. It is desirable to produce the BCG mycobacteria in high yields for manufacture of the BCG vaccine. Currently, vaccine components are made using many hundreds or even thousands of individual flasks. This is highly inefficient, with batch to batch variation, but continuous culture methods or large scale fermenter culture are not available to replace this inefficient method.

An object of the present invention is to provide for batch or continuous culture of mycobacteria, in particular continuous culture that will maintain bacterial virulence and provide cells of defined and consistent properties. A further object is to provide a growth medium for culture of mycobacteria.

Accordingly, a first aspect of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation and in the presence of sufficient detergent so that a substantially homogenous suspension of cells is maintained.

Preferably, the method of the invention comprises growing said mycobacteria in batch or continuous culture, at a temperature of 35°C +/- 10°C, at a

dissolved oxygen tension of at least 1.0 percent, at a pH of 6.9 +/- 0.9 and with agitation in the presence of sufficient detergent to maintain a substantially homogenous suspension of single cells.

In use of the present invention, illustrated by specific embodiments described below in more detail, and using *Mycobacteria tuberculosis*, we have developed a method which allows high yields of bacteria from both batch and continuous culture systems. Further, we have shown that mycobacteria generated using the methods of embodiments of the present inventions are highly virulent as demonstrated in a standard guinea pig infection model of *M.tuberculosis*. Indeed, potency of *Mycobacteria tuberculosis* grown using these methods is comparable with *M.tuberculosis* grown using the solid agar slope method.

In a specific embodiment of the present invention, growth of *M.tuberculosis* in steady-state continuous culture achieved a biomass yield of $1.2gl^{-1}$ cell dry weight. Cells grown in this continuous culture, and also in batch culture, displayed virulence comparable to cells grown on Middlebrook agar slopes, strongly indicating the suitability of these methods for growth of mycobacteria spp, such as *M. tuberculosis* or *M. bovis*, for prolonged periods in chemostat culture.

It is thus an advantage that the method of the invention enables growth at increased cell densities and with reduced mean generation times, or doubling times. It is further of advantage that using the method of the present invention expression of virulence determinants has been maintained. Thus, the method is of application for production of mycobacteria such as for incorporation into BCG vaccines.

In the present invention, the term "batch culture" is used in its conventional sense to refer to a fixed volume of culture medium which is inoculated with a micro-organism. After a period of adjustment, termed the lag phase, the organism starts to grow and multiply reaching the maximum growth rate

possible in that environment – termed exponential growth. After multiple generations essential nutrients become depleted or toxic metabolites build-up causing growth to slow and eventually cease. This is a closed system and the environment is constantly changing as the organism grows. This type of culture is typically performed in small shake flasks, 50 – 500 ml, where only temperature is controlled though in embodiments of the invention temperature, pH and oxygen have been controlled. A further benefit of methods of the invention is that through control of environmental parameters there is reduced batch-to-batch variation, leading to cultures of move consistent composition and less bacterial heterogeneity, which is a significant consideration during production of vaccine components from these cultures.

The term "fermenter culture" is similarly used with reference to a type of batch culture operated with more control over the environmental parameters such as pH and aeration. Fermenters are normally used for production, hence the culture volume is larger.

The term "continuous culture" is used to refer typically to a culture of constant volume to which medium is added continuously and from which there is continuous removal of any overflow culture. By adding growth components in the fresh medium, the organism continues to multiply. When this system reaches equilibrium, cell number and nutrient status remain constant, it is said to be in steady state.

Lastly, the term "chemostat culture" refers to the current most common type of continuous-culture-device. Two elements are generally used to control the culture, the concentration of an essential nutrient, such as carbon source, and the flow rate. After inoculation the culture grows until an essential nutrient becomes depleted and limits growth, however, the continuous addition of fresh medium containing the limiting nutrient permits continued growth. The cell density is controlled by the concentration of limiting nutrient added. The limiting nutrient can be altered by manipulating the medium formulation. The

rate of medium addition controls the growth rate (generation time) of the culture.

In the method of the invention, it is preferred that the culture temperature is maintained at 35°C +/- 10°C, more preferably 35°C +/- 5°C, and in specific embodiments of the invention this preferred temperature has been maintained for in excess of three weeks with continuous mycobacteria growth. The pH of the culture medium, in continuous operation, is typically controlled to within +/- 0.9 of pH 6.9, more preferably +/- 0.5 of pH 6.9. pH may be controlled using addition of acid or alkaline solution to the culture medium, according to the pH correction required. In specific embodiments of the invention described below, sodium hydroxide at a concentration of 0.5M and sulphuric acid at a concentration of 0.5M is used. The dissolved oxygen concentration of the culture is typically at an initial level of at least 40% (v/v) air saturation, preferably at least 50%.

Detergent is present in the method of the invention as a dispersing agent to maintain a high proportion of the mycobacteria suspended in a substantially homogenous suspension, preferably as single cells or small clumps containing 2 to 10 bacilli. Once thus dispersed, cells can grow in an environment enabling higher growth rates under relatively constant and controlled conditions. It is possible, though the applicant does not wish to be bound by any theory, that once mycobacteria form pellicles as in previous culture methods they can not thereafter be dispersed - the invention may thus improve the previous methods by preventing or reducing loss of bacilli into such pellicles. Some detergents in use slowly release toxic components into the culture medium, so the amount of detergent present should not be so high as to risk the detergent or any of its components reaching toxic levels. Similarly, excess detergent can lead to foaming of the culture and should be avoided. The level of detergent may suitably be at least 0.1% (v/v).

Anionic detergents are preferred, in particular esters of sorbitan and derivatives

thereof, though it is believed that the advantageous effects of the invention and the results obtained in the specific embodiments may likewise be realised using any of a wide range of detergents. Particularly good results have been obtained using a polyethane-diyl derivative of a sorbitan ester, namely Tween 80, other such esters being Tween 20, Tween 40 and Tween 60. Despite the presence of detergent it has been found that albumin may be omitted from the growth medium without slowing mycobacterial growth.

In use of the methods of the invention for batch culture of mycobacteria, detergent may be present at from 0.1 to 1.0 % (v/v), more preferably from 0.1 to 0.5 %, most preferably about 0.2 % (v/v).

In use of the methods of the invention for continuous culture of mycobacteria, detergent may be present at least 0.1 % (v/v), more preferably at least 0.15 % (v/v), and most preferably about 0.2%, its level further preferably being no more than 1.0%, and usually no more than 0.7%. When the culture is being operated continuously, medium is continuously introduced into the culture, the rate of introduction expressed as a dilution rate. The culture of the invention can be carried out continuously with a dilution rate of at least 0.02 h^{-1}, resulting in a high yield of bacteria, and these bacteria have been found to have preserved their virulence. A dilution rate of at least 0.025 h^{-1} can also be sustained, and in a specific embodiment a dilution rate of about 0.03 h^{-1} was achieved in continuous culture, representing a mean doubling time of about 24 hours.

The invention further provides a growth medium for culture of mycobacteria, comprising:-

a carbon source;

a mitogen;

trace elements comprising at least Mg, K, P and S;

a nitrogen source.

The carbon source is preferably selected from glucose, glycerol and an amino acid, and combinations of these carbon sources. The mitogen is present to induce cell division and is preferably asparagine, though other mitogens from inorganic sources are also suitable. Trace elements in the growth medium are preferably selected from Ca, Mg, Zn, Co, Cu, Mn, Ni, Fe, K, and mixtures thereof, and the nitrogen source is selected from an amino acid and an ammonium salt.

The growth medium optionally further comprises an amino acid component selected from alanine, arginine, asparagine, asphaltic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and mixtures thereof. The amino acid component can contribute the nitrogen source in the medium.

Other optional components are a vitamin/co-factor component selected from inositol, thiamine, calcium pantothenate, co-enzyme A, nicotinamide, biotin, DL-thiocitic acid, and mixtures thereof, and one or more components selected from sodium hydroxide, glutathione, glycerol, haemin, sodium pyruvate and *a*-ketoglutarate.

Thus, a particularly preferred embodiment of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation in the presence of sufficient detergent so that a substantially homogenous suspension of single cells is maintained, and in the presence of a growth medium according to combination of the above-described media.

The present invention is now described with reference to the following specific embodiments, illustrated by drawings in which:-

Fig. 1 shows a schematic view of continuous culture apparatus

according to the invention;

Fig. 2 shows a graph of optical density at 540 before and after initiation of continuous culture;

Fig. 3 shows viable *M. tuberculosis* in guinea pig lungs following aerosol challenge with bacteria grown using the medium of the invention; and

Fig. 4 shows viable *M. tuberculosis* in guinea pig spleens following aerosol challenge with bacteria grown using the medium of the invention.

Referring to Fig. 1, a medium reservoir 1 is attached via medium addition pump and line 2 to culture vessel 6. The glass culture vessel 6 comprises a titanium toplate through which are connected temperature probe 7, oxygen electrode 8, air inlet and sparger 9, vent 10, pH electrode 11, alkali addition line 12 and acid addition line 13. Samples of the content of the culture vessel may be taken through sample port 14 and effluent from the culture vessel drains into or is pumped into effluent reservoir 15.

This continuous culture apparatus is used for continuous culture of mycobacteria as described in examples below.

Example 1

Materials and Methods

Strain

Studies was performed with *M. tuberculosis* strain H37Rv (NCTC cat. no. 7416) - a representative strain of *M.tuberculosis*. Stock cultures were grown on Middlebrook 7H10 + OADC for 3 weeks at 37 ± 2°C harvested and stored at -70°C as a dense suspension in deionised water.

Culture Medium

A chemically defined culture medium was developed, and was designated CAMR mycobacterial Medium (see Appendix 1 below). The medium was prepared with high quality water from a Millipore water purification system and filter sterilised by passage through a 0.07 μm pore size cellulose acetate membrane filter capsule (Sartorius Ltd). Middlebrook 7H10 + OADC agar was used to prepare inoculum cultures, enumerate the number of culturable bacteria in chemostat samples, and to assess culture purity.

Culture apparatus

Culture experiments were performed in a one litre glass vessel operated at a working volume of 500 ml. The culture was agitated by a magnetic bar placed in the culture vessel coupled to a magnetic stirrer positioned beneath the vessel. Culture conditions were continuously monitored and controlled by an Anglicon Microlab Fermentation System (Brighton Systems, Newhaven), linked to sensor probes inserted into the culture through sealed ports in the top plate. The oxygen concentration was monitored with a galvanic oxygen electrode (Uniprobe, Cardiff) and was controlled through feedback control of the agitation rate. Temperature was monitored by an Anglicon temperature probe, and maintained by a heating pad positioned beneath the culture vessel. Culture pH was measured using an Ingold pH electrode (Mettler-Toledo, Leicester) and controlled by automatic addition of either sodium hydroxide (0.5 M) or sulphuric acid (0.5 M). For continuous culture, the culture system was operated by controlling nutrient addition from the medium reservoir and a constant culture volume was maintained by an overflow tube fitted to the side of the vessel.

Inoculation and culture

The vessel was filled with 350 ml of sterile culture medium and parameters were allowed to stabilise at 37°C ± 2°C, pH 6.9 ± 0.2 and a dissolved oxygen tension of approximately 70% air saturation. A dense inoculum suspension was prepared by resuspending Middlebrook agar cultures, grown

at 37 ± 2°C for 3 week, in sterile deionised water. The inoculum was aseptically transferred to the culture vessel, to provide an initial culture turbidity of approximately 0.25 at 540 nm. After inoculation the culture was allowed to establish for approximately 50 h. As the culture entered exponential growth, a further 100 ml medium was added and batch growth was monitored by optical density and viable count determination.

For continuous culture, the culture was inoculated and allowed to establish for approximately 50 h as detailed. The culture was then operated in fed batch mode for 48 h with medium addition (approx. 100 ml) as the culture entered exponential growth and 24 h later. Continuous culture was then initiated at a dilution rate of 0.03 h^{-1} [equivalent to a mean generation time (MGT) of 24 h]. Culture parameters were maintained at a dissolved oxygen tension of 50 % (v/v) air saturation at 37 ± 2°C and pH 6.9 ± 0.2. Growth was monitored by optical density, dry weight and viable count determination.

Culture analyses

The optical density of culture samples was recorded at 540 nm (OD_{540}) in a UV-260 spectrophotometer (Pye Unicam) against a water reference. Culture biomass was determined by dry weight analysis. Samples were treated with 4% (v/v) formaldehyde for at least 24 h and filtered through a pre-dried, pre-weighed, 0.45 μm pore sized, nylon membrane filter (Gelman Sciences), under vacuum. The membrane was rinsed with 10 ml of deionised water, before re-drying to a constant weight, and re-weighing.

Total viable counts were performed by preparing a 10-fold dilution series of the sample in sterile deionised water, and plating 100μl aliquots of appropriate dilutions onto Middlebrook 7H10 plates in triplicate. The plates were incubated at 37°C for 3 weeks before enumerating the number of colonies formed. Culture purity was checked by plating neat samples onto Middlebrook 7H10 and Blood agar and incubating at 37°C.

Results

Batch culture

Growth of *M. tuberculosis* strain H37Rv was established in CAMR Mycobacteria medium supplemented with 0.2% Tween 80. After inoculation the culture followed typical batch growth kinetics with a lag phase of approximately 50 hours before entering exponential growth. A minimum doubling time of approximately 14 h was recorded. Cultures were predominantly single cell suspensions.

Continuous culture

Steady-state growth, at a MGT of 24 h, was normally reached 10 days after initiation of continuous culture. Cultures were dense suspensions containing approximately 5×10^8 cfu ml^{-1} and a biomass yield of approximately 1.2 gl^{-1} cell dry weight. Cells were short rods 2 to 3 μm long with occasional clumps of up to 20 cells. Glycerol, the principal carbon source was not depleted during steady state growth, with a residual concentration of 1.25 gl^{-1}. Tween 80 was present in an amount of 0.1% and enabled the growth of *M. tuberculosis* in a homogeneous suspension made up substantially of single cells at a growth rate conducive to chemostat culture. Cultures grown in the absence of Tween 80 formed large clumps and surface pellicles and continuous culture was not possible.

Other observations made during operation of this culture indicate that for long-term continuous production it may be necessary to clean the vessel or preferably transfer to a clean vessel at regular intervals, say every 5-6 weeks. Mycobacteria can sometimes tend to attach to the vessel wall, impeding continuous culture, and we have also found, separately, that lowering the oxygen tension to at least 20% air saturation assists to counter this problem.

Influence of culture mode on virulence

The virulence of batch and chemostat grown cells was compared with cells grown on Middlebrook agar. Guinea pigs challenged with plate-grown cells

produced a classical disease process with exponential multiplication in guinea pig lungs up to three weeks post infection, when lung counts reached 10^6 to 10^7 c.f.u. per lung (fig. 3). Low numbers of bacilli were detected in spleen tissues 2 weeks post infection followed by an exponential increase up to day 21, after which growth rate declined (fig. 4). Infection with both batch and chemostat grown cells produced a comparable disease process demonstrating that culture virulence was retained.

The invention thus provides methods for batch and continuous culture of dispersed mycobacteria in high yield and without loss of virulence, and also provides a growth medium therefor.

In cultures of the invention, large-scale and consistent production of vaccine components is enabled, for manufacture eg of bacterial subunits, whole bacilli for vaccine uses and whole bacilli for immune therapies.

References

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Wayne, L. G. 1977. Synchronised replication of *Mycobacterium tuberculosis*. Infect. Immun. 17:528-530.

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Appendix 1

Composition of CAMR MYCOBACTERIA MEDIUM

Stock solutions.	mg l^{-1}	Stock Solutions	mg l^{-1}
	Amino acids		
L- alanine	100	L-leucine	100
L-arginine	100	L-lysine	100
L-asparagine	2000	L-methionine	100
L-aspartic acid	100	L-phenylalanine	100
L-cysteine	500	L-proline	100
L-glutamine	100	L-serine	100
L-glutamic acid	100	L-threonine	100
L-glycine	100	L-tryptophan	100
L-histidine HCl	100	L-tyrosine	50
L-isoleucine	100	L-valine	100
	Inorganic salts		
$CaCl_2 . 2H_2O$	0.55	$NaMO_4 \cdot 2H_2O$	1.2
$MgSO_4 . 7H_2O$	214	$NiSO_4 \cdot 6H_2O$	0.53
NH_4VO_3	1.2	$FeSO_4 \cdot 7H_2O$	10
$ZnSO_4 \cdot 7 H_2O$	28.75	KH_2PO_4	220
$CoCl_2 \cdot 6H_2O$	0.48	Na_2SO_4	150
$CuSO_4 \cdot 5H_2O$	0.025	KOH	56
$MnCl_2 \cdot 4H_2O$	0.02		
	Vitamins and co-factors		
inositol	2	nicotinamide	1
thiamine HCl	2	biotin	0.1
calcium -pantothenate	2	DL-thiocitic acid	0.1
coenzyme A	0.1		
	Other		
ACES buffer	10000	haemin	2.0
$NaHCO_3$	42	sodium pyruvate	1000
glutathione (reduced)	500	α-ketoglutarate	1000
glycerol	2 ml	Tween 80	2.0 ml

Stock solution formulations.

(CAMR MYCOBACTERIA MEDIUM).

Stock solutions.	g l^{-1}	mg l^{-1}
Solution 2.		
$CaCl_2 . 2H_2O$		55.5
$MgSO_4 . 7H_2O$	21.4	
NH_4VO_3		117
$ZnSO_4 \cdot 7 H_2O$	2.875	
Solution 3.		
$CoCl_2 \cdot 6H_2O$		47.6
$CuSO_4 \cdot 5H_2O$		2.5
$MnCl_2 \cdot 4H_2O$		2.0
$NaMO_4 \cdot 2H_2O$		121
$NiSO_4 \cdot 6H_2O$		52.6
conc. HCl		0.5 ml
Solution 4.		
$FeSO_4 \cdot 7H_2O$	1.0	
Conc. HCl		0.5 ml
Solution 5.		
L- alanine	1.0	
L-arginine	1.0	
L-asparagine	20.0	
L-aspartic acid	1.0	
L-glutamine	1.0	
L-glutamic acid	1.0	
L-glycine	1.0	
L-histidine HCl	1.0	
L-isoleucine	1.0	
L-leucine	1.0	
L-lysine	1.0	
L-methionine	1.0	
L-phenylalanine	1.0	
L-proline	1.0	
L-serine	1.0	
L-threonine	1.0	
L-tryptophan	1.0	
L-valine	1.0	

Stock Solutions	g l^{-1}	mg l^{-1}
Solution 6.		
sodium pyruvate	100	
Solution 7.		
α-ketoglutarate	100	
Solution 8.		
inositol		200
thiamine HCl		200
calcium - pantothenate		200
nicotinamide		100
biotin		10
Solution 9.		
DL-thiocitic acid	1.0	
ethanol		950 ml
Solution 10.		
coenzyme A	1.0	
Solution 11.		
haemin	2.0	
KOH	56	

All solutions were prepared with high quality Millipore water.

Preparation of CAMR MYCOBACTERIA MEDIUM.

Solution	Quantity
ACES buffer	10.0 g
KH_2PO_4	0.22 g
Na_2SO_4	0.15 g
Millipore water	500 ml
Solution 2	10 ml
Solution 3	10 ml
Solution 5	100 ml
Solution 6	10 ml
Solution 7	10 ml
Solution 8	10 ml
Solution 9	0.1 ml
Solution 10	0.1 ml
L-cysteine HCl	0.5 g
Glutathione (reduced)	0.5 g
L-tyrosine	0.05 g
$NaHCO_3$	0.042 g
Glycerol	0.2 ml
Solution 4	10 ml
Adjust pH to 6.5 with 20% KOH	
Solution 11	1 ml
Tween 80	2.0 ml
Millipore water up to 1 litre	

Filter sterilise by passage through 0.07 μm filter (Sartorius Ltd.)

CLAIMS

1. A method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation and in the presence of sufficient detergent so that a substantially homogenous suspension of cells is maintained.

2. A method according to Claim 1, comprising culturing the mycobacteria in the presence of at least 0.1% (v/v) detergent.

3. A method according to Claim 1 or 2, comprising culturing the mycobacteria at a temperature of 35°C +/- 10°C.

4. A method according to any of Claims 1 to 3, comprising maintaining the pH at 6.9 +/- 0.9.

5. A method according to any of Claims 1 to 4, comprising culturing the mycobacteria with an initial dissolved oxygen concentration of at least 1% (v/v) air saturation.

6. A method according to any of Claims 1 to 5, for culture of mycobacteria selected from *M. tuberculosis*, *M. bovis* and *M. vaccae*.

7. A method according to any of Claims 1 to 6 for batch culture of mycobacteria, wherein detergent is present at from 0.1 to 1.0 % (v/v).

8. A method according to Claim 7, wherein detergent is present at about 0.2 % (v/v).

9. A method according to any of Claims 1 to 6 for continuous culture of mycobacteria, wherein detergent is present at at least 0.1 % (v/v).

10. A method according to Claim 9, wherein detergent is present at at least 0.15 % (v/v).

11. A method according to Claim 9 or 10, wherein the culture is carried out continuously with a dilution rate of at least 0.02 h^{-1}.

12. A method according to Claim 11, wherein the culture is carried out continuously with a dilution rate of at least 0.025 h^{-1}.

13. A method of culture of mycobacteria, comprising growing said mycobacteria in continuous culture, at a temperature of 35°C +/- 10°C, at a dissolved oxygen tension of at least 1 percent, at a pH of 6.9 +/- 0.9, at a dilution rate of at least 0.02 h^{-1} and with agitation in the presence of sufficient detergent to maintain a substantially homogenous suspension of single cells.

14. A growth medium for culture of mycobacteria, comprising:-
a carbon source;
a mitogen;
trace elements comprising at least Mg, K, P and S;
a nitrogen source.

15. A growth medium according to Claim 14, wherein the carbon source is selected from glucose, glycerol and an amino acid.

16. A growth medium according to Claim 14 or 15, wherein the mitogen is asparagine.

17. A growth medium according to any of Claims 14 to 16, comprising trace elements selected from Ca, Mg, Zn, Co, Cu, Mn, Ni, Fe, K, and mixtures thereof.

18. A growth medium according to any of Claims 14 to 17, wherein the

nitrogen source is selected from an amino acid and an ammonium salt.

19. A growth medium according to Claim 18, comprising an amino acid component selected from alanine, arginine, asparagine, asphaltic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and mixtures thereof.

20. A growth medium according to any of Claims 14 to 19, further comprising a vitamin/co-factor component selected from inositol, thiamine, calcium pantothenate, co-enzyme A, nicotinamide, biotin, DL-thiocitic acid, and mixtures thereof.

21. A medium according to any of Claims 14 to 20, further comprising one or more components selected from sodium hydroxide, glutathione, glycerol, haemin, sodium pyruvate and α-ketoglutarate.

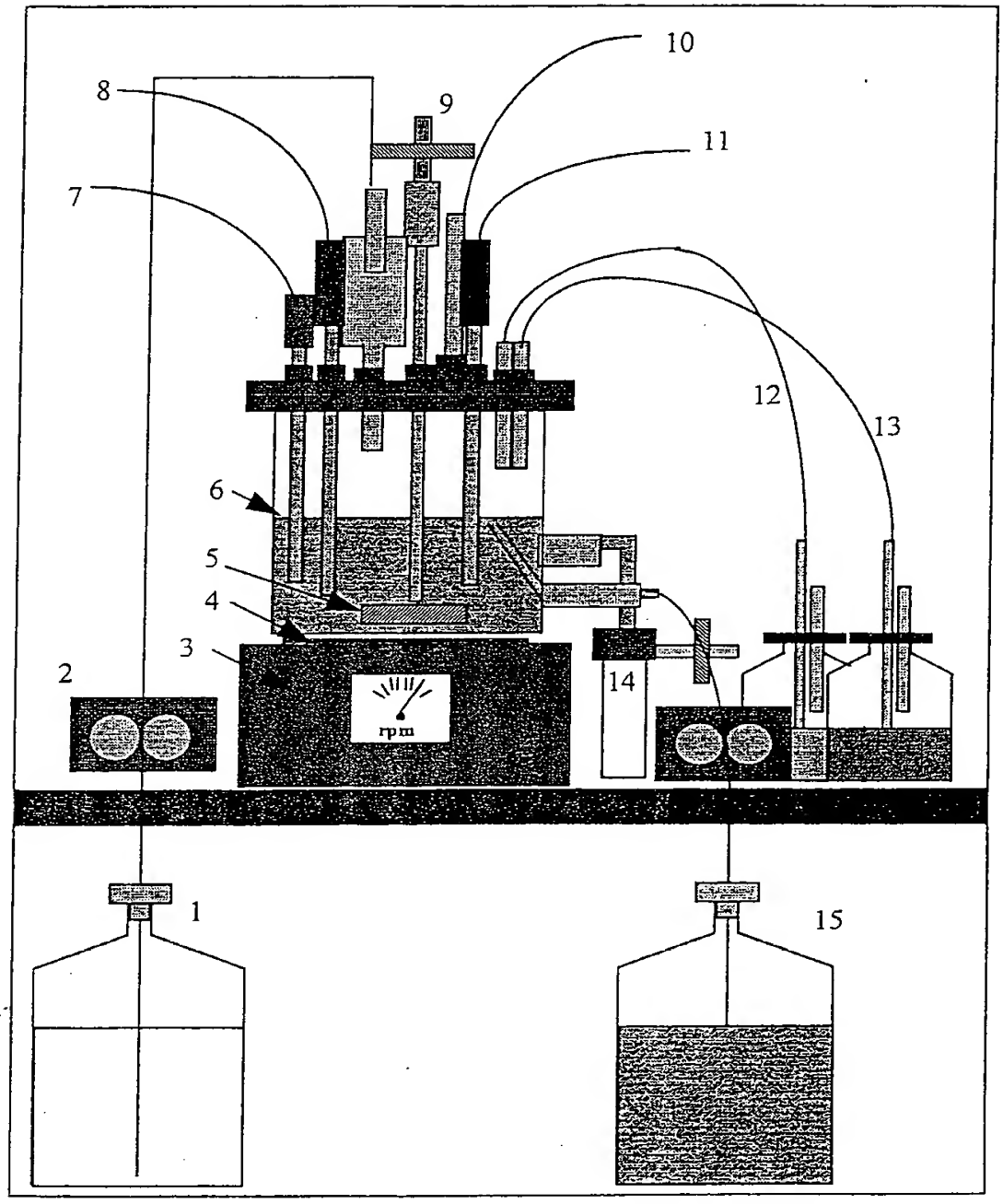
22. A method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation in the presence of sufficient detergent so that a substantially homogenous suspension of single cells is maintained, and in the presence of a growth medium according to any of Claims 14 to 22.

23. A method of culture of mycobacteria substantially as hereinbefore described with reference to the examples.

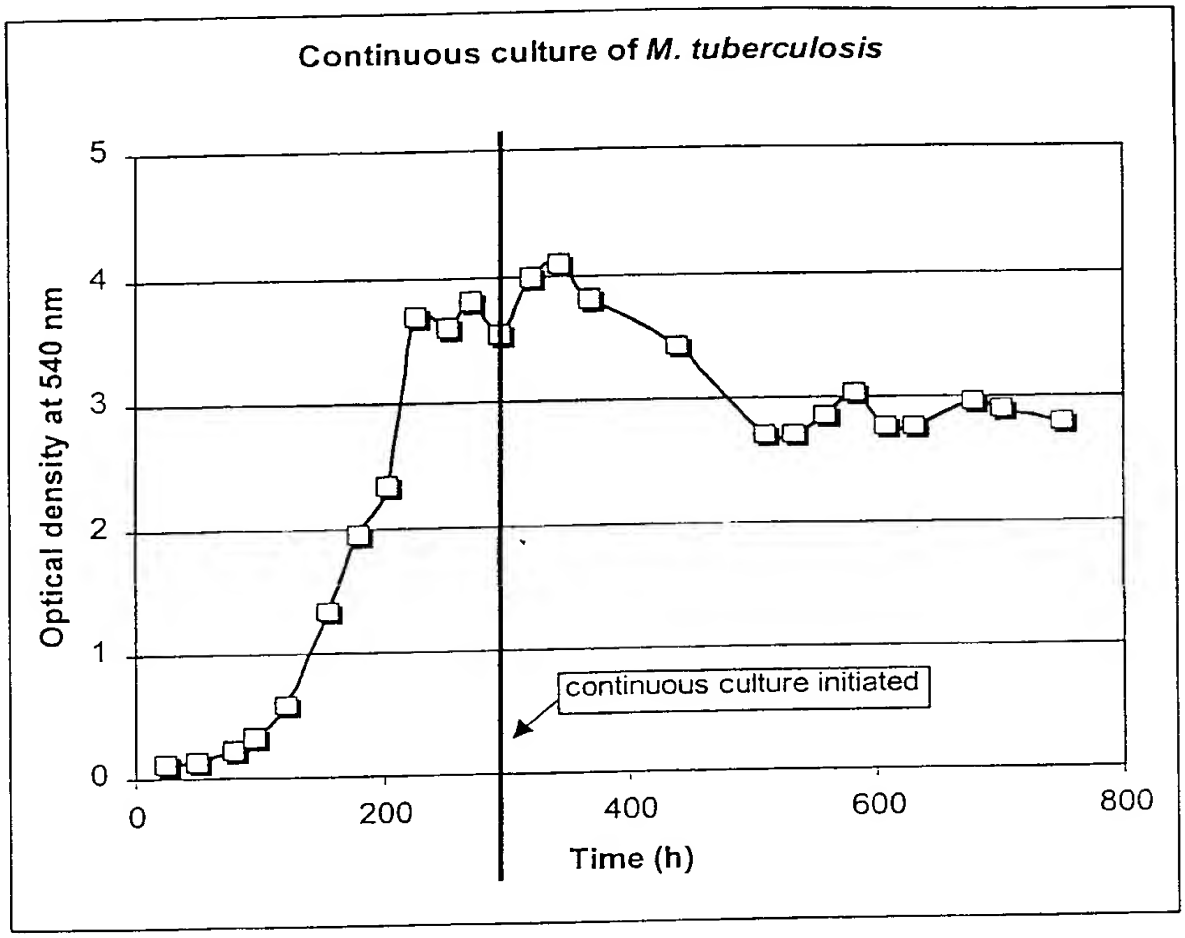
24. A growth medium substantially as hereinbefore described with reference to the examples.

FIGURE 1

SCHEMATIC OF THE CONTINUOUS CULTURE APPARATUS



(1) medium reservoir; (2) medium addition pump and line; (3) magnetic stirrer unit; (4) heating pad; (5) magnetic bar; (6) culture vessel with titanium top plate; (7) temperature probe; (8) oxygen electrode; (9) air inlet and sparger; (10) vent; (11) pH electrode; (12) alkali addition; (13) acid addition; (14) sample port; (15) effluent reservoir.



After inoculation the culture was operated in batch for 4 days. Medium addition was then initiated in fed-batch mode. Continuous medium addition was started at 300 h.

Fig. 2

Virulence Assessment

Influence of culture mode on the virulence of *M. tuberculosis*: The virulence of chemostat grown cells was compared with cells grown to mid-exponential batch phase in ABCD ModTB medium and on Middlebrook agar. Guinea pig challenge with plate-grown cells produced a classical disease process with exponential multiplication in guinea pig lungs up to three weeks post infection, when lung counts reached 10^6 to 10^7 c.f.u. per lung. After 3 weeks the lung counts declined marginally (Figure 1a). Low numbers of bacilli were detected in spleen tissues 2 weeks post infection followed by an exponential increase up to day 21 (Figure 1b). Infection with both batch and chemostat grown cells produced a comparable disease process demonstrating that culture virulence was retained (Figure 1).

a + b

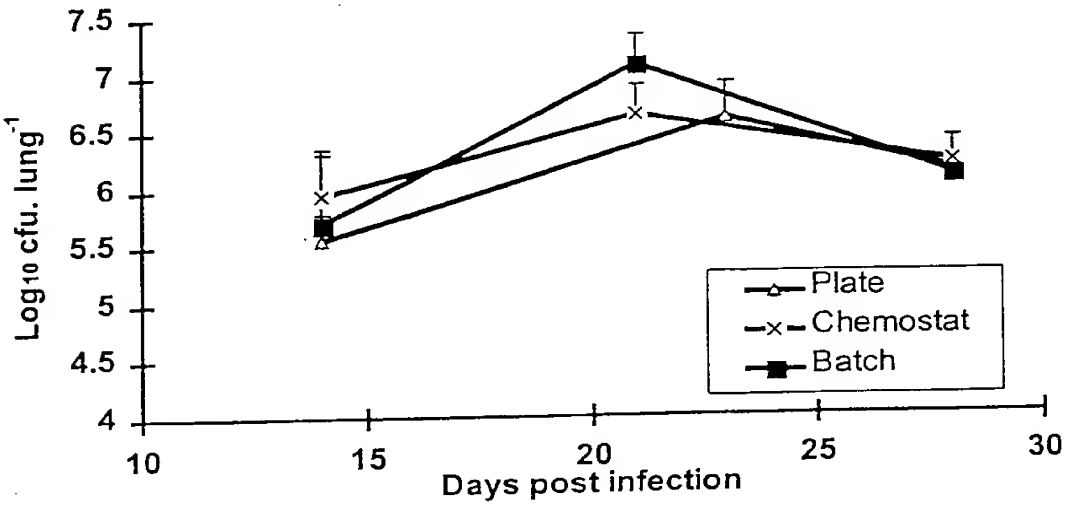


Figure 3 Viable *M. tuberculosis* in guinea pig lungs following aerosol challenge; error bars + standard deviation.

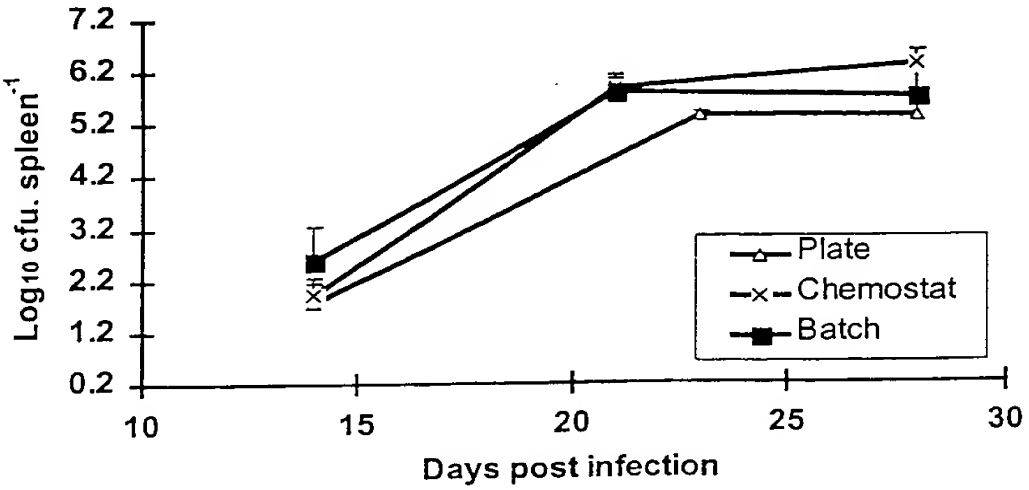


Figure 4. Viable *M. tuberculosis* in guinea pig spleens following aerosol challenge; error bars + standard deviation.

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